

Mutagenic and epigenetic effects of DNA methylation

Mark L. Gonzalgo, Peter A. Jones *

Department of Biochemistry and Molecular Biology, Urologic Cancer Research Laboratory, USC / Norris Comprehensive Cancer Center, University of Southern California, School of Medicine, Los Angeles, CA 90033, USA

Abstract

Tumorigenesis begins with the disregulated growth of an abnormal cell that has acquired the ability to divide more rapidly than its normal counterparts (Nowell, P.C. (1976) *Science*, 194, 23–28 [1]). Alterations in global levels and regional changes in the patterns of DNA methylation are among the earliest and most frequent events known to occur in human cancers (Feinberg and Vogelstein (1983) *Nature*, 301, 89–92 [2]); Gama-Sosa, M.A. et al. (1983) *Nucleic Acids Res.*, 11, 6883–6894 [3]; Jones, P.A. (1986) *Cancer Res.*, 46, 461–466 [4]). These changes in methylation may impair the proper expression and/or function of cell-cycle regulatory genes and thus confer a selective growth advantage to affected cells. Developments in the field of cancer research over the past few years have led to an increased understanding of the role DNA methylation may play in tumorigenesis. Many of these studies have investigated two major mechanisms by which DNA methylation may lead to aberrant cell cycle control: (1) through the generation of transition mutations via deamination-driven events resulting in the inactivation of tumor suppressor genes, or (2) by altering levels of gene expression through epigenetic effects at CpG islands. The mechanisms by which the normal function of growth regulatory genes may become affected by the mutagenic and epigenetic properties of DNA methylation will be discussed in the framework of recent discoveries in the field.

Keywords: DNA methylation; Transition mutation; DNA methyltransferase; Tumor suppressor gene; p53; p16

1. Introduction

The covalent modification of the C-5 position of cytosine by DNA (cytosine-5) methyltransferase (MTase) resulting in the formation of 5-methylcytosine (5-mCyt) gives this base unique properties. This enzymatic conversion is the only epigenetic modification of DNA known to exist in vertebrates and is essential for normal embryonic development [5,6].

Methylation of cytosine occurs predominantly at the CpG palindrome in vertebrate DNA, but recent studies have shown that mammalian cells also possess the ability to methylate CpNpG sites in transfected plasmid DNA sequences [7]. The presence of 5-mCyt at CpG dinucleotides has resulted in the 5-fold depletion of this sequence in the genome during the course of vertebrate evolution [8], probably due to spontaneous deamination of 5-mCyt → T or enzyme-induced deamination of C → U [9–11].

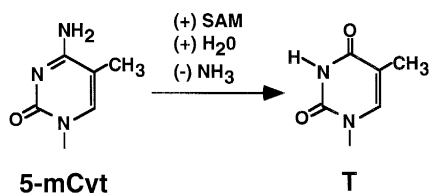
Certain areas of the genome, however, do not show such suppression and are referred to as ‘CpG islands’ [12,13]. These regions comprise about 1% of

* Corresponding author. Tel.: +1 (213) 764 0816; Fax: +1 (213) 764 0102.

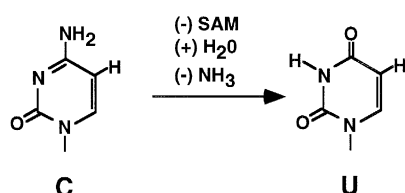
Possible Contribution of DNA Methylation to Cancer

A. Mutagenic Effects

1. Spontaneous deamination of 5-mCyt \rightarrow T

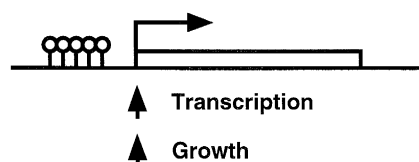


2. Enzyme-mediated deamination of C \rightarrow U



B. Epigenetic Effects

1. Hypomethylation of proto-oncogenes



2. Hypermethylation of tumor suppressor genes

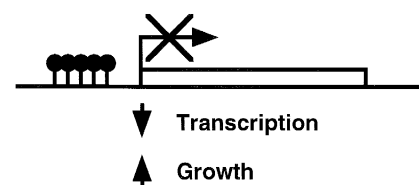


Fig. 1. Possible contribution of DNA methylation to cancer. (A) Mutagenic effects of DNA methylation by either (1) spontaneous deamination of 5-mCyt \rightarrow T, or (2) enzymatic deamination of C \rightarrow U under conditions in which AdoMet is limiting. (B) Epigenetic effects of CpG island methylation on gene expression. Lollipops indicate CpGs that are unmethylated (open) or methylated (closed).

vertebrate genomes, yet account for approximately 15% of the total number of CpG dinucleotides. These areas contain the expected frequency of CpGs and remain unmethylated in the germline [12]. CpG islands are typically between 0.2–1 kb in length and are located upstream of many housekeeping and tissue specific genes, but may also extend into gene coding regions. The methylation of cytosines within CpG islands in somatic tissues is believed to affect gene function by altering transcription [14]. Hypermethylation of CpG islands results in transcriptional inactivation, while hypomethylation leads to the potential for gene activity. Hypermethylation as a mechanism of gene silencing is known to occur in normal cells only on transcriptionally inactive genes on the X chromosome [15] and in parentally imprinted genes [16,17]. Such epigenetic effects of 5-mCyt may be important in tumorigenesis as it relates to the abnormal activation of proto-oncogenes

or inactivation of tumor suppressor genes (see Fig. 1).

2. Mutational properties of 5-methylcytosine

Methylation of cytosine residues was first demonstrated to be mutagenic in *E. coli* nearly two decades ago [18]. These initial studies identified methylated cytosines as hotspots for spontaneous base substitutions. Mutations which occur at CpG dinucleotides in vertebrate DNA can be attributed to the hydrolytic deamination of 5-mCyt and are easily recognized because of the nature of base substitutions. Deamination of 5-mCyt at CpG dinucleotides results in the formation of TpG. Alternatively, if deamination occurs on the complementary DNA strand CpA is generated. The conversion of 5-mCyt to T is be-

lieved to be the result of endogenous mutagenic processes rather than mutagenesis caused by exogenous factors [19]. Methylation of cytosine at a CpG dinucleotide increases the probability of a C → T or corresponding G → A transition mutation between 12- and 42-fold [10,20,21].

The increased deamination rate of 5-mCyt relative to C, however, still does not account for the high frequency of mutagenesis observed at CpG sites [22]. Differences in the repair efficiencies of premutagenic lesions may be partly responsible for this discrepancy. The G:T mispairs resulting from deamination of 5-mCyt are believed to be more difficult for the cell to repair than G:U mispairs which can result from the deamination of cytosine, since thymine unlike uracil is a normal component of DNA. The high efficiency of repair of G:U but not G:T mismatches by the well characterized uracil-DNA glycosylase (UDG) enzyme [23] may also contribute to the increased frequency of mutagenesis caused by 5-mCyt deamination [24,25]. Excision of U has been found to be as much as 6000-fold more efficient than excision of T at identical template sites using extracts from human colonic mucosa [25].

3. Estimated rates for spontaneous deamination of 5-mCyt

The chemical rate for the deamination of 5-mCyt in DNA has been determined to gain further insight into the contribution of the mutagenic properties of this base to human disease. The rates for hydrolytic deamination of 5-mCyt to thymine at 37°C in vitro have been calculated in single-stranded ($9.5 \times 10^{-10} \text{ s}^{-1}$) and double-stranded DNA ($5.8 \times 10^{-13} \text{ s}^{-1}$) [22,26]. While these in vitro studies also demonstrated approximately a 5- and 2-fold higher deamination rate of 5-mCyt compared to C in single- and double-stranded DNA, respectively, these values are still too low to account for the high frequency of mutations observed at CpGs.

Several studies have been performed to determine the in vivo rate of 5-mCyt deamination. The in vivo rate was originally calculated to be approximately $1.7 \times 10^{-16} \text{ s}^{-1}$ based on extrapolation of in vitro 5-mCyt deamination rates combined with the estimated error frequency of the G:T mismatch repair

system [27]. Another study of spontaneous germline mutations in the factor IX gene in patients with hemophilia B calculated the in vivo mutation rate at CpG to be approximately $5.8 \times 10^{-17} \text{ s}^{-1}$ for a generation time of 20 years [28]. This estimate was based on a comparison of CpG mutation frequencies with other types of mutations in the factor IX gene and extrapolation of the rate of CpG mutation from these values.

The in vivo rate of CpG mutation has been recently calculated in *Alu* repetitive elements within the *p53* tumor suppressor gene in the primate germline [21]. *Alu* sequences are repetitive elements approximately 300 bp in length which are found throughout primate genomes and are initially CpG rich [29]. CpG dinucleotides within *Alu* elements can become methylated after transposition and C → T mutations which are the hallmark of 5-mCyt deamination may be generated. The rate of 5-mCyt deamination in vivo was estimated to be between $1.5\text{--}4.5 \times 10^{-16} \text{ s}^{-1}$ by establishing the time of insertion of *Alu* elements within the *p53* gene via evolutionary lineages and by using the unique signature of mutations at CpGs [21]. This in vivo mutation rate is at least 1250-fold slower than the in vitro chemical rate of 5-mCyt deamination in double-stranded DNA confirming earlier studies and suggesting that current estimates of mismatch repair at CpG dinucleotides may be underestimated.

4. Enzyme-mediated deamination of cytosine to uracil

Although 5-mCyt is inherently mutagenic by virtue of its ability to generate C → T transitions through spontaneous deamination, an alternate pathway leading to C → U mutations via covalent interaction of the MTase with its target cytosine base has been demonstrated in bacteria using a sensitive genetic reversion assay [11,30]. The bacterial *HpaII* MTase caused an approximately 10^4 -fold increase in the rate of C → U transition mutations in the absence of AdoMet in this experimental system. The normal reaction mechanism for cytosine methylation proceeds with the MTase first binding to the C-6 position of the target base by nucleophilic attack, thus generating a covalent intermediate [31]. A

methyl group is then added to the C-5 position of cytosine eliminating a C-5 proton followed by release of the covalent intermediate. Enzyme-facilitated deamination of cytosine may be significant under conditions in which *S*-adenosylmethionine (AdoMet) is limiting. These conditions may prolong the half-lives of reaction intermediates while destabilizing the amino group at the C-4 position of cytosine and thereby increasing the probability of C → U deamination [11,32]. The resulting C → U transitions are then propagated as C → T mutations at CpG dinucleotides if they are not repaired before DNA replication. Recently, it has been shown that a DNA MTase can also interact with 5-mCyt to directly cause deamination of 5-mCyt → T [33].

Further experiments in our laboratory have demonstrated that mutator enzymes can be derived from DNA MTases when the cofactor binding activity of the enzyme is impaired [30]. Mutant *HpaII* methylases were created by altering the conserved FXGXG motif of the AdoMet binding pocket. Expression of the mutant methylases resulted in the generation of C → U transitions at DNA methylation sites in bacteria even in the presence of normal levels of AdoMet, thus providing support for the model of enzyme-mediated deamination. In addition, the G:U mispairs generated by the mutant enzymes in *E. coli* which possessed UDG were found to be inefficiently repaired resulting in a mutator phenotype characteristic of the C → U → T pathway.

Greater insight into the mechanism of enzyme-induced deamination by DNA MTases was provided by the determination of the crystal structure of the *HhaI* methylase interacting with its target base [34]. The normal cytosine base was substituted with 5-fluorocytosine to trap the enzyme revealing that the target base was completely flipped out of the DNA helix into the active site of the enzyme. A possible explanation for the inefficient repair of G:U mispairs was elucidated by studies demonstrating that bacterial MTases such as *HhaI* and *HpaII* not only bind to DNA mismatches more tightly than their normal target sequences, but are also capable of blocking repair of these mismatches [35,36]. Both *HhaI* and *HpaII* enzymes also retained methyl transfer ability upon binding to G:U mismatches which resulted in methylation of the C-5 position of U creating T, albeit at low frequencies.

Methyltransferases interact with G:T and G:U mismatches with higher affinities than for the normal G:C targets, suggesting that DNA binding of MTases is inversely correlated with the stabilities of the target base pairs. Analysis of the enzyme-DNA complexes revealed that dissociation constants (K_d) for the binding of *M. HhaI* to G:T and G:U mismatches were approximately 5- and 10-fold less than those for the G:C targets, respectively [36]. The abilities of the MTases to bind these premutagenic lesions and to block DNA repair in vitro was determined by incubating a plasmid containing a G:U mismatch with increasing concentrations of *M. HhaI* prior to incubation with UDG. These studies suggest additional mechanisms by which a DNA MTase could contribute to mutagenesis even in the presence of a highly active UDG repair system. However, the actual contribution of mismatch repair blockage by the MTase or enzyme-mediated deamination pathways to the increased mutability of CpG sites in mammals remains to be demonstrated.

The significance of enzyme-mediated deamination to mutagenesis in prokaryotic and eukaryotic organisms is not known. Experiments performed by our laboratory to investigate the possible roles of DNA methylation in colon carcinogenesis have shown that although levels of MTase mRNA expression are slightly elevated in colon tumors compared to adjacent normal colonic mucosa, these differences represent on average only a 4-fold increase in MTase expression [37]. Similar levels of increased MTase enzyme activity on the order of 3.4-fold in colon tumors compared to surrounding normal epithelium have also been observed [38]. One study, however, has reported levels of MTase expression to be increased as high as 200-fold in colon tumors compared to tissue from patients with benign lesions [39]. The observation of increased MTase expression must be interpreted with caution, since MTase expression is cell-cycle dependent [40] and any increase in expression of this gene observed in tumors may simply reflect a higher proportion of dividing cells or increased nuclear to cytoplasmic ratio compared to the normal cell population.

In addition to differences in MTase expression between normal and tumor colonic mucosa, differences in AdoMet levels and sequence analysis of conserved domains of the human MTase cDNA have

also been studied as possible factors which may contribute to enzyme-facilitated deamination of cytosine in tumor cells [37]. Ion-pair HPLC analysis of the concentrations of AdoMet and its breakdown product *S*-adenosylhomocysteine (AdoHcy) in extracts of colon carcinoma tissues and adjacent normal colonic mucosa were measured. No significant differences between the AdoMet/AdoHcy ratio in tumors versus normal colonic mucosa were observed, suggesting that AdoMet is not limiting for the normal methylation of DNA by MTase in the later stages of human colon tumor development. Furthermore, no mutations in the MTase cDNA coding for the AdoMet binding pocket have been found [37], thus downplaying the possible contribution of enzymatic deamination of C → U in colon carcinogenesis. The most likely mechanism for the high preponderance of mutations at CpGs is probably the result of spontaneous deamination of 5-mCyt combined with inefficient repair of the resulting premutagenic DNA mismatches [25]. However, the involvement of the MTase enzyme-mediated pathway for C → U → T mutations in colon cancer cannot be completely ruled out.

5. DNA methylation and mutational patterns in tumor suppressor genes

Mutations in the *p53* tumor suppressor gene are believed to occur in more than half of all solid human tumors [41]. The types of mutations which are present in the *p53* gene and other growth regulatory genes may lead to an understanding of factors which contribute to mutagenesis. Approximately 24% of point mutations in *p53* which have been observed in human cancers are C → T transitions at CpG dinucleotides [41]. Interestingly, five of six mutational hotspots found in the most evolutionarily conserved regions of the *p53* gene are located at CpGs, and transitions occurring at these sites are consistent with 5-mCyt deamination. We and others have previously demonstrated that all of the mutational hotspots in the *p53* gene occurring at CpGs are methylated [19,42,43], thus suggesting an involvement of 5-mCyt as an endogenous mutagen.

The high frequency of mutations at CpG sites within *p53* appears to play a significant role in

some, but not all types of human cancers (see Fig. 2). Approximately 37% of the mutations in various internal cancers are consistent with deamination-driven events at CpG dinucleotides, however, only 11% of mutations found in lung cancer can be attributed to 5-mCyt deamination at CpGs [44]. Differences in the mutational spectra of *p53* in these types of cancers suggest that endogenous 5-mCyt deamination may play a larger role in the genesis of mutations found in cancers such as bladder, breast and colon, while oxygen radicals and exogenous substances such as benzo[*a*]pyrene found in tobacco smoke may be responsible for the high number of transversions observed in lung cancer [41].

Analysis of the mutational spectra of the *p16*/CDKN2/MTS1 tumor suppressor gene in a variety of tumors also suggests that the relative involvement of endogenous or exogenous factors may lead to specific patterns of mutation associated with particular cancer types. Both intragenic deletions and point mutations have been observed in melanoma cell lines not containing homozygous deletions of *p16* [45,46]. Mutational screening of several melanoma cancer cell lines revealed a high number of CC → TT tandem mutations and C → T transitions at dipyrimidine sites [47]. These types of alterations are characteristic of UV-induced mutations which promote covalent linkages between two adjacent pyrimidine bases. Characterization of the alterations in the *p16* gene in patients with familial melanoma revealed very few C → T transitions at CpGs, suggesting that at least for melanoma, exogenous factors such as UV radiation rather than endogenous methylation-induced mutations play a more predominant role in altering gene function. Comparison of the genetic alterations in *p16* found in melanoma cell lines and tumors with internal cancers reveals differences in the types and patterns of mutations, suggesting that endogenous factors such as 5-mCyt deamination at CpGs may be more relevant to the genesis of internal cancers [48].

The spectrum of mutations in the *p53* gene in the germline contrasts significantly with that of the *p16* gene (see Fig. 2). While 5-mCyt deamination at CpGs appears to play a major role in generating C → T germline mutations in *p53*, this is not the case for *p16* germline mutations. The mutational pattern of *p16* in the germline indicates a relatively

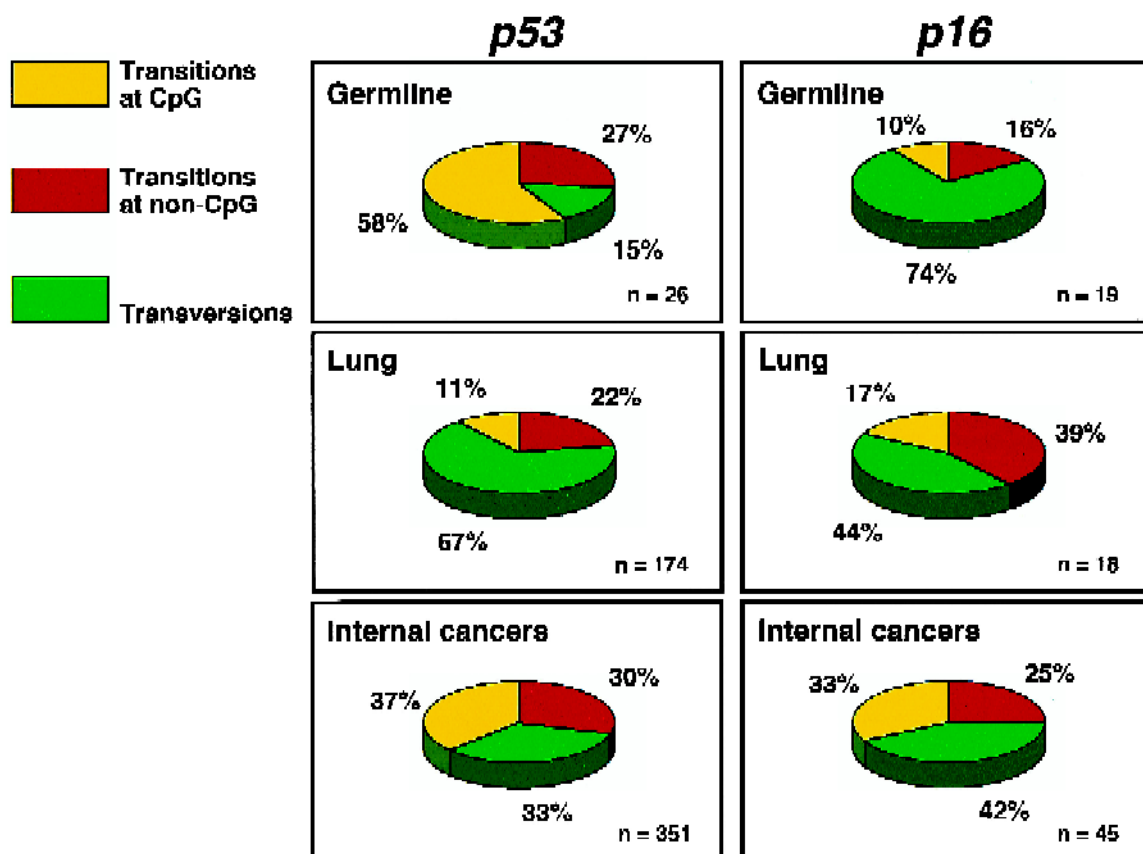


Fig. 2. Comparison of mutations between the *p53* and *p16* tumor suppressor genes in the germline and primary tumors. Transitions at CpGs presumably resulting from 5-mCyt deamination account for the majority of germline *p53* mutations while transversions account for the majority of *p16* germline mutations. The mutational patterns of both genes in lung cancer are believed to be caused by exposure to exogenous compounds. Transitions at CpGs are observed in approximately one-third of internal cancers for both *p53* and *p16*. The percent frequency of CpG dinucleotides within the sequence for reported mutations is: 4% for *p53* and 12% for *p16*. Internal cancers for *p53* are: bladder, breast, and colon. Internal cancers for *p16* are: bile duct, bladder, breast, esophagus, head/neck, gall bladder, and pancreas. Percentages were calculated from reported mutations for *p53* [44] and *p16* [48]. Percentages of *p16* germline mutations are as follows if possible founder-effects are considered and multiple mutations at individual sites are excluded ($n = 12$): 17%, transitions at CpG; 25%, transitions at non-CpG; 58%, transversions.

low contribution of 5-mCyt deamination-induced transitions compared to *p53* germline mutations. The presence of CpG islands within the coding regions of *p16* may actually protect these sequences from deamination-induced mutational events since CpG islands are normally unmethylated. Interestingly, a higher frequency of C \rightarrow T transitions at CpGs in the *p16* gene is observed in various somatic internal cancers. This finding suggests that CpGs within *p16* may have undergone de novo methylation in somatic cells and that at least for some cancers, 5-mCyt

deamination may play an increased role in mutagenesis.

Although these data must be interpreted with caution due to the small sample size of internal tumors harboring intragenic *p16* mutations, it is interesting to propose alternative models for the involvement of DNA methylation and its effects on growth regulatory genes during tumor development. In the case of *p53*, methylation of CpGs is present in sperm [19], thus predisposing this gene to deamination-driven mutations from early in development.

The *p16* gene, however, appears to be unmethylated in the germline and only in certain types of cancers does it become abnormally methylated resulting in an increased frequency of C → T transitions at CpG dinucleotides [49].

While analysis of the mutational spectra of genes provides insight into the contribution of 5-mCyt deamination to mutagenesis, recent experiments using knockout mice heterozygous for the DNA MTase gene combined with administration of 5-aza-2'-deoxycytidine (5-aza-CdR) have led to an increased recognition of the possible roles for DNA methylation in the early stages of tumor development [50]. *Apc^{Min}* mice have a germline point mutation in the *Apc* tumor suppressor gene which causes the formation of hundreds of intestinal polyps during the first 6 months of life. Treatment of *Apc^{Min}* mice heterozygous for the DNA MTase gene with 5-aza-CdR resulted in substantially decreased polyp formation compared to control *Apc^{Min}* mice receiving no drug. While the exact mechanisms underlying decreased polyp formation in the presence of DNA hypomethylation are unclear, Laird et al. [50] suggest that the mutagenic effects of the MTase rather than increased proto-oncogene expression via an epigenetic pathway may be responsible for the development of colonic neoplasia.

6. Epigenetic effects of DNA methylation

The epigenetic properties of DNA methylation are heritable and unlike the mutagenic effects of 5-mCyt do not involve alterations of the primary DNA sequence. Methylation of cytosine residues contained within CpG islands of certain genes has been inversely correlated with gene activity, but it is still unclear whether this methylation is actually responsible for causing different activity states of a gene or is merely the result of such changes. Recent experiments, however, provide much evidence to support a causal relationship between DNA methylation and effects on transcriptional activity. Methylation at CpG islands may lead to decreased gene expression by a variety of mechanisms including: disruption of local chromatin structure, inhibition of transcription factor-DNA binding, or by recruitment of proteins which interact specifically with methylated sequences indi-

rectly preventing transcription factor binding [5,14,51].

Many studies have demonstrated an inverse correlation between methylation of CpG islands and gene expression, but most CpG islands on autosomal genes remain unmethylated in the germline and methylation of these islands is usually independent of gene expression. Tissue-specific genes are typically unmethylated in the respective target organs but are methylated in the germline and in non-expressing adult tissues, while CpG islands of constitutively expressed housekeeping genes are normally unmethylated in the germline and in somatic tissues. Decreased levels of global hypomethylation are common findings in tumorigenesis [2,3,52]. Experimental manipulation of the methylation status of CpG islands has primarily been studied using demethylating agents to determine the epigenetic relationship between DNA hypomethylation and gene expression.

In contrast to CpG islands on autosomal genes, methylation of CpG islands has been demonstrated for several genes on the inactive X-chromosome and is associated with transcriptional silencing [53,54]. Analysis of the promoter of the human phosphoglycerate kinase-1 (*PGK-1*) gene, which is located in the middle of a CpG island revealed no methylation on the active X chromosome and extensive methylation of the CpG island on the inactive X chromosome [55]. Several transcription factors were also observed to bind only those DNA sequences which were unmethylated in the *PGK-1* promoter, indicating that methylation at CpGs is capable of inhibiting protein-DNA binding. Interestingly, treatment of hamster-human cell hybrids containing an inactive human X-chromosome with 5-azacytidine (5-aza-CR) caused reactivation of the *PGK-1* gene [56]. Expression of *PGK-1* was associated with demethylation of a large region within the CpG island, suggesting that a specific zone of unmethylated DNA in the promoter was necessary for transcription.

Increased methylation and heterochromatinization of CpG islands has been proposed as a mechanism for silencing the expression of non-essential genes during the establishment of immortal cell lines [57,58]. Experiments in our laboratory have shown that the CpG island of the *MyoD1* gene becomes increasingly methylated and heterochromatinized during immortalization of 10T1/2 cells [59]. Further

analysis of the *MyoD1* CpG island in primary bladder and rhabdomyosarcoma tumors demonstrated that several sites were hypermethylated, thus supporting a possible *in vivo* function for methylation of this gene. Our laboratory has also investigated the methylation status of various genes following treatment of 10T1/2 cell lines with 5-aza-CdR. Several CpG sites within four genes: α_1 -globin, *DHFR*, *GFAP*, and *MyoD1* were extensively demethylated after drug treatment [60]. Although extensive demethylation was not always associated with differences in levels of gene expression, altered chromatin structure was observed in α_1 -globin and *MyoD1* following 5-aza-CdR treatment. Increased nuclease sensitivity of these regions demonstrated that induction of transcriptionally competent chromatin structures was associated with DNA hypomethylation.

A Chinese hamster cell line (G12) system has recently been used to study the expression and chromatin changes of a bacterial xanthine guanine phosphoribosyl transferase (*gpt*) transgene and the role of DNA hypermethylation and compaction of chromatin structure as a mechanism for decreasing gene expression [61]. Treatment of the G12 cell line with various carcinogenic nickel compounds resulted in high levels of 6-thioguanine (6TG) resistant colonies indicative of *gpt* inactivation. Interestingly, silencing of *gpt* expression was associated with hypermethylation and increased heterochromatinization of the *gpt* locus. Reactivation of *gpt* expression was induced following treatment of 6TG-resistant clones with 5-aza-CR and was associated with both hypomethylation and chromatin decondensation. These studies provide further insight into the mechanisms by which DNA methylation may alter gene expression and also demonstrate a new model for gene silencing by carcinogens.

7. Sites within CpG islands may regulate gene expression

Certain CpG sites within the promoter region of genes may function as transcriptional control centers which are affected by DNA methylation. The mouse metallothionein I (mMTI) promoter has been methylated at various CpG dinucleotides *in vitro*, resulting in decreased activity in transient transfection

experiments [62]. Inhibition of promoter activity, however, correlated specifically with methylation of CpGs located at the preinitiation domain and not at other sites within the mMTI promoter. Further experiments support an indirect model for suppression of promoter activity by methyl-binding proteins. Methylation of all CpG sites within the mMTI promoter using an *in vitro* assay system had no effect on the binding of transcription factors TFIIA and TFIID to the TATA box region, but transcription of the methylated promoter was significantly reduced *in vivo* [62]. These studies suggest that the density of methylated CpG sites in certain areas of the promoter such as the preinitiation domain are more important for transcriptional control than flanking sequences, and that certain proteins such as MeCP1 and MeCP2 [5,63,64] may bind to these methylated sequences thus inhibiting formation of the preinitiation complex. Additionally, levels of CpG methylation as low as 7% have been shown to inhibit gene expression by as much as 67–90% in an episomal system based on the Epstein-Barr virus (EBV) in a human embryonic kidney cell line [65]. Methylation of all CpG sites within the EBV-based plasmid resulted in no detectable levels of gene expression.

An alternative model for methylation-induced gene silencing is based upon the importance of individually methylated CpGs within the promoter rather than CpG density [66]. Differential methylation at a single CpG site within the Epstein-Barr Virus (EBV) latency C promoter may be critical for transcriptional activation. Interaction of a protein with cellular binding activity (CBF2) with the EBNA-2 response region was inhibited by methylation at a single CpG dinucleotide. Treatment of cell lines with 5-aza-CR caused demethylation of several sites within the EBV genome and also induced CAT reporter gene expression. Thus, methylation at a single CpG site in the EBNA-2 response region of the EBC latency C promoter was shown to affect binding of a protein necessary for transcriptional activation.

8. Transcriptional silencing of tumor suppressor genes

Abnormal methylation of the CpG islands associated with tumor suppressor genes may also cause

Table 1
Methylation of CpG islands in putative tumor suppressor genes

Cancer type (gene)	% Tumors methylated	Ref.
Retinoblastoma (<i>Rb</i>)	8	Greger et al. [67] Sakai et al. [68]
Renal cell carcinoma (<i>VHL</i>)	19	Herman et al. [71]
Wilms' tumor (<i>H19</i>)	67	Steenman et al. [78]
Internal cancers ^a (<i>p16</i>)	33	Gonzalez-Zulueta et al. [49] Herman et al. [71] Merlo et al. [75]

^a Internal cancers include: bladder, breast, colon, lung, head and neck.

decreased gene expression (see Table 1). Increased methylation of such regions may lead to progressive reduction of normal gene expression resulting in the selection of a population of cells with a selective growth advantage. The first report of an epigenetic change involving hypermethylation of a CpG island in somatic cells was described for the retinoblastoma (*Rb*) gene [67]. This gene was found to be hypermethylated in a sporadic unilateral retinoblastoma tumor among 21 tumors examined. Additional studies have also reported hypermethylation of *Rb* in 5 out of 56 retinoblastoma cases [68]. Although the patient sample sizes for these studies were small and *Rb* expression in tumors was not reported, in vitro methylation of additional sites within the *Rb* CpG island has been shown to inactivate the *Rb* promoter [69].

Inactivation of the von Hippel-Lindau (*VHL*) gene by mutation or allelic loss has been implicated to be the initiating event in spontaneous cases of clear-cell renal carcinoma [70,71]. Abnormal methylation of the CpG island in the 5' region of *VHL* may provide yet another mechanism for loss of normal gene function. Herman et al. [71] found hypermethylation of the *VHL* CpG island and lack of expression in 19% of clear-cell renal carcinomas containing no intragenic mutations. Interestingly, treatment of a renal cell culture line with 5-aza-CdR caused demethylation and expression of a previously silent *VHL* gene. These experiments provide further evidence for a causal relationship between these processes.

A higher rate of mutations and homozygous deletions of *p16* has been reported in a variety of cultured tumor cells compared to primary tumors [72,73]. De novo methylation of the 5' CpG island of *p16* has also been shown to occur in many different

cancer types [49,74], and associated transcriptional gene silencing may account for the decreased frequency of *p16* mutations previously reported for primary tumors. 5-aza-CdR treatment of lung cancer cell lines demonstrating *p16* 5' CpG island methylation without gene expression resulted in partial demethylation of the 5' CpG island and transcriptional activation of the gene [75]. Preliminary experiments in our laboratory suggest that treatment of bladder cancer cell lines with 5-aza-CdR results not only in demethylation and subsequent expression of *p16*, but is also accompanied by restoration of growth control [76].

Methylation of CpG islands as a plausible mechanism for gene silencing has also been observed in parentally imprinted genes [16,17]. The human insulin-like growth factor II (*Igf-2*) and *H19* genes are imprinted in the human and demonstrate expression of the paternal *Igf-2* and maternal *H19* alleles. Expression of *H19* in Wilms' tumor and rhabdomyosarcoma cell lines resulted in reduced growth, indicating possible tumor suppressor function for this gene [77]. It has been recently shown that the promoter of the *H19* gene becomes abnormally methylated and is associated with decreased expression in some cases of Wilms' tumor [78]. The maternal chromosome reverted to a paternal pattern of expression which included expression of *Igf-2*, methylation of the *H19* promoter, and lack of *H19* expression.

9. Conclusions

Methylation of cytosines at CpG dinucleotides has both mutational and epigenetic consequences on mammalian genomes. Spontaneous deamination of

5-mCyt \rightarrow T or enzymatic deamination of C \rightarrow U are two major pathways by which DNA methylation may contribute to mutagenesis. Comparison of the mutational spectra between different genes and tumors will help in understanding the mutagenic effects of DNA methylation in various types of cancers. The presence of methylated cytosines in the germline may predispose some genes to a higher frequency of deamination-induced transitions. Other genes, however, may be protected from these types of mutations because of the existence of CpG islands within their coding regions. CpG islands associated with such genes may subsequently undergo abnormal de novo methylation in somatic tissues resulting in an increased frequency of 5-mCyt \rightarrow T mutations. The epigenetic effects of DNA methylation and their relationship to the transcriptional silencing of growth regulatory genes are other important considerations in defining key processes involved in tumorigenesis. The application of drugs known to alter methylation patterns in order to reactivate tumor suppressor genes silenced by CpG island methylation may also be an avenue for future therapeutic options.

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